

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 997 529 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
03.05.2000 Bulletin 2000/18

(51) Int Cl.7: **C12N 15/49**, C12N 7/00,
C12N 7/04, C12N 5/10,
A61K 39/21, C07K 14/155,
C07K 16/10

(21) Application number: **99306508.5**

(22) Date of filing: **18.08.1999**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(72) Inventors:
• **Deng, Ruitang**
Old Lyme, Connecticut 06371 (US)
• **Sheppard, Michael George**
North Stonington, Connecticut 06359 (US)

(30) Priority: **24.08.1998 US 97645 P**

(83) Declaration under Rule 28(4) EPC (expert solution)

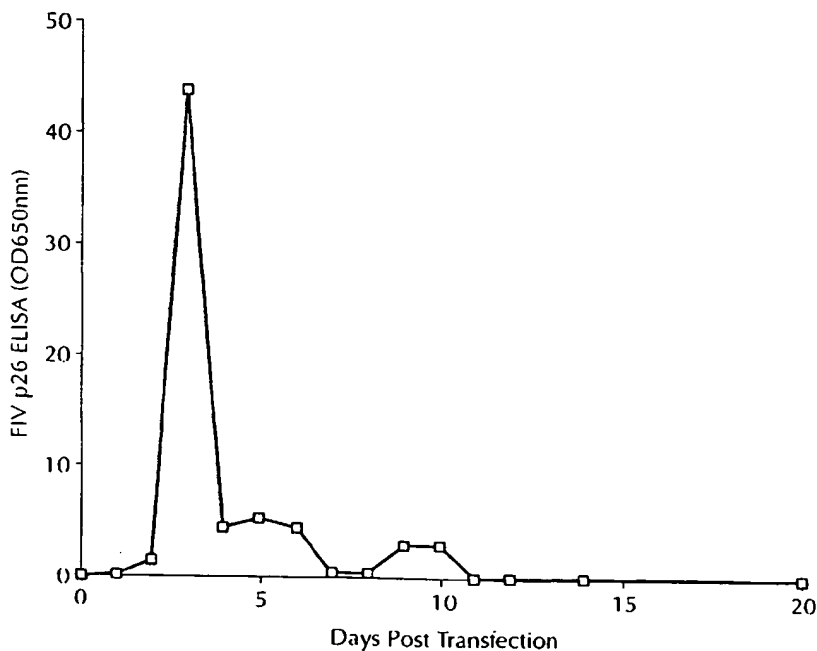
(74) Representative:
Simpson, Alison Elizabeth Fraser et al
Urquhart-Dykes & Lord,
91 Wimpole Street
London W1M 8AH (GB)

(71) Applicant: **Pfizer Products Inc.**
Groton, Connecticut 06340 (US)

(54) Feline immunodeficiency virus strain FIV-141 and uses thereof

(57) The present invention is directed to a novel strain of feline immunodeficiency virus, designated herein as FIV-141, and to attenuated forms of the virus

produced by mutating specific regions of the viral genome. The virus and mutated forms of the virus may be used to induce the production of antibodies to FIV-141, and in vaccines designed to protect cats from FIV.

**FIG. 1****EP 0 997 529 A2**

A. Compositions and Methods Based Upon the FIV-141 Virus

[0009] In its first aspect, the present invention is directed to a substantially purified FIV-141 virus having a genomic sequence corresponding to that of SEQ ID NO:1, to host cells infected with the virus and to progeny virus produced in the host cells. The term "substantially purified" means that FIV-141 has been separated from all other strains of virus and, particularly, from all other strains of FIV. Host cells that may be used for growing virus include peripheral blood mononuclear cells (PBMCs). Progeny virus may be isolated using standard procedures as discussed below.

[0010] The FIV-141 virus and host cells infected with the virus can be used to infect animals for the purpose of inducing the production of antibodies that react preferentially with one or more strains of FIV. "Preferential binding" of antibodies, as used herein, refers to an antibody having at least a 100-fold greater affinity for FIV than for any other virus or non-FIV protein. Antibodies may be generated in any of the animals commonly used for this purpose (such as, e.g., mice, rabbits, goats, or sheep) but, preferably, antibodies will be made in domestic cats. When virus is used to induce antibody production, it may, if desired, be inactivated prior to infection. Inactivation procedures may involve treating the virus with formalin, paraformaldehyde, phenol, lactopropionate, ultraviolet light, heat, psorlens, platinum complexes, ozone or other viricidal agents. When host cells expressing FIV-141 are used to induce antibody production, the cells may be fixed prior to infection. Typically, this will involve treating the cells with paraformaldehyde as described herein, but other methods may also be employed. Antibodies made to FIV-141 are themselves included within the scope of the invention and may be purified using techniques well known in the art (see, e.g., Harlow *et al.*, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.).

[0011] In another aspect, the invention is directed to a whole virus vaccine comprising inactivated FIV-141 virus. An immune response may be induced in a cat by administering this vaccine at a dosage and for a duration sufficient to induce protective immunity against subsequent infection with FIV-141. Typically, the vaccine will be administered parenterally with two or more inoculations being given at intervals of, e.g., two to eight weeks. The invention also includes a fixed cell vaccine, which is comprised of a host cell infected with the FIV-141 virus. Administration of this vaccine will follow the same general procedures as used for the whole virus vaccine. Standard procedures well known in the art may be used to optimize immunization protocols.

B. Compositions and Methods Based Upon FIV-141 Genomic Nucleic Acid

[0012] In another aspect, the present invention is directed to a substantially purified nucleic acid molecule (DNA or RNA) having a sequence corresponding to that of SEQ ID NO:1. As used in this context, "substantially purified" means that the desired product is essentially free from contaminating cellular components. A "substantially pure" nucleic acid will typically comprise at least 85% of a sample, with greater percentages being preferred. Contaminants may include proteins, carbohydrates or lipids. One method for determining the purity of a nucleic acid is by electrophoresing a preparation in a matrix such as polyacrylamide or agarose. Purity is evidenced by the appearance of a single band after staining. Other methods for assessing purity include chromatography and analytical centrifugation. The FIV-141 nucleic acid may be used in place of the whole virus to transfect host cells and to thereby induce the production of progeny virus or viral proteins.

[0013] The invention also encompasses methods of inducing the production of antibodies to FIV-141 by injecting nucleic acid directly into an animal or by administering host cells transfected with the nucleic acid. As with the procedures discussed above in connection with the whole virus, host cells may be fixed prior to administration. Antibodies may be purified from animals and used in assays designed to detect the presence of FIV in culture medium or in a biological fluid.

[0014] Host cells transfected with FIV-141 genomic DNA may also be used in a vaccine for immunizing cats. If desired, such cells may be fixed to reduce viral infectivity, e.g., by treatment with an agent such as paraformaldehyde. Vaccines made in this manner may be used to induce an immune response in a cat. The vaccine may be administered using a standard immunization protocol optimized for the induction of protective immunity against subsequent infection with FIV-141 or, if desired, some other strain of FIV.

C. Attenuated FIV-141 Virus and Vaccines

[0015] Before a whole virus can be administered to an animal as a vaccine, it must be converted into a non-pathogenic form. As discussed above, this may be accomplished by inactivating the virus or fixing host cells. An alternative method involves introducing mutations into the virus to transform it into an attenuated form. The phrase "attenuated virus" as used in this context, refers to a virus that has substantially reduced infectivity compared to its wild type counterpart. Infectivity may be measured in PBMCs, as described in the Examples section herein below.

[0016] Thus, the invention is directed to an attenuated FIV-141 virus that exhibits significantly reduced infectivity for feline T lymphocytes relative to the wild type (*i.e.*, non-mutated) virus. The attenuated virus is produced by mutating

invention should be administered at a dosage and for a duration sufficient to reduce one or more clinical signs and viral load associated with the infection of the mammal. When the lentivirus is a strain of FIV, the mammal treated will be a cat and the signs associated with the infection will include immunological abnormalities such as an abnormally low level of CD4⁺ T-lymphocytes or an abnormally elevated number of CD8⁺ T-lymphocytes. Other clinical signs will typically include alopecia, anemia, chronic rhinitis, conjunctivitis, diarrhea, emaciation, enteritis, gingivitis, hematochezia, neurological abnormalities and dermatitis.

[0024] The attenuated lentivirus (e.g., an attenuated strain of FIV), or host cells infected with such a virus, may be used in vaccine at a concentration sufficient to induce immunity when administered to a mammal (e.g., a cat). An immune response may then be induced by administering such a vaccine at a dosage and for a duration sufficient to induce protective immunity against subsequent infection by at least one strain of lentivirus.

F. Methods of Making and Using Mutated Lentivirus Nucleic Acid

[0025] The present invention is also directed to a method of producing a nucleic acid suitable for use in a vaccine against lentivirus, e.g. FIV, infection. This is accomplished by reverse transcribing the genomic RNA of the lentivirus; cloning the reverse transcript; mutating one or more genes selected from the group consisting of MA, CA, NC, SU, TMf, ORF(2), CT, ENV, Vif, Vifn, Vifc, V3/4, V7/8, IN, DU, and RRE; and then cloning the mutated nucleic acid. Preferably, mutations should be such that, upon introduction into a host cell, an attenuated virus is made that has significantly reduced infectivity relative to lentivirus produced by the unmutated, wild type nucleic acid. In the case of FIV, infectivity should be reduced or eliminated for feline T-lymphocytes.

[0026] Mutated lentivirus nucleic acid may be purified and used to transfect host cells, make progeny virus, and make antibody in the same way as described above for FIV-141. In addition, the nucleic acid, or host cells transfected with the nucleic acid, may be incorporated into a vaccine and used to induce an protective immunity in a mammal. Preferably, the nucleic acid will encode an attenuated strain of FIV that has significantly reduced infectivity in feline PBMCs, including T-lymphocytes such as FeP2 cells. Under these circumstances, the immune response will be induced in a cat.

Brief Description of the Drawings

[0027] Figure 1: FIV Production from Transfected Cells. Crandell Feline Kidney (CRFK) cells were transfected with a plasmid comprising the full length FIV-141 genome. Beginning at 24 hours post-transfection, cell supernatants were harvested and assayed for the presence of FIV p26 capsid protein using an enzyme immunoassay.

[0028] Figure 2: Infection of FeP2 T Lymphocytes by Co-culture. CRFK cells were grown in six well plates and transfected with plasmid DNA encoding the full length FIV-141 genome. Forty-eight hours after transfection, 2×10^6 FeP2 cells were introduced into each well. Beginning 72 hours after co-cultivation, FeP2 cells were separated and their supernatants were tested for the presence of FIV p26 capsid protein by ELISA.

[0029] Figure 3: Infection of FeP2 Cells by Adsorption. 2×10^6 FeP2 cells were suspended in 200 ul of FIV-141 virus-containing conditioned medium derived from CRFK cells transfected with the full length infectious FIV-141 clone. Beginning at four days post-infection, FeP2 cell supernatants were tested for the presence of FIV-141 virus using a p26 ELISA assay.

[0030] Figure 4: Expression of FIV-141 Viral Protein by Deletion Clones. A variety of clones mutated to delete FIV genes or regulatory regions were made and transfected into CRFK cells. After 48 hours, cell supernatants were assayed for the presence of FIV p26 capsid protein by ELISA. The results for each deletion clone, as well as the wild type FIV-141 molecular clone, are shown.

[0031] Figures 5-10: Infection of FeP2 T Lymphocytes by FIV-141 Mutants. CRFK cells were grown in six well plates and infected with one of three different FIV-141 deletion clones: TMf del, ENV del, or NC del. After 48 hours, FeP2 cells were added to each well and co-cultures were maintained for an additional 72 hours. The FeP2 cells were then separated from the CRFK cells and assayed for the presence of p26 antigen using an ELISA assay. Monitoring of p26 levels was repeated every 34 days and results are shown in Figure 5. The experiment was repeated using: Vifn del, Vifc del and Vif del (Figure 6); MA del and CA del (Figure 7); V3/4 del, V7/8 del and CT del (Figure 8); ORF(2) del (Figure 9); and DU del, SU del, IN del, and RRE del (Figure 10).

Detailed Description of the Invention

A. Production of FIV-141 and DNA Encoding the Virus

[0032] The present invention is directed to a novel strain of feline immunodeficiency virus (designated herein "FIV-141") that is distinguished from all similar strains based upon its genomic nucleic acid sequence and biological functions.

that have been introduced may be found in Table 1.

Table 1

Mutations in FIV-141		
MUTATION	NUCLEOTIDES DELETED	AMINO ACIDS DELETED
MA del	123 bases, nucleotides 879-1001	41 amino acids, residues 85-125 at the C-terminus of the MA protein
CA del	114 bases, nucleotides 1056-1169	38 amino acids, residues 9-46 at the N-terminus of the CA protein
NC del	242 bases, nucleotides 1635-1876	21 amino acids in the CA region and 51 amino acids in the NC region, accompanied by a reading frame shift preventing expression of the terminal portion of the NC protein
ENV del	2103 bases deleted, nucleotides 6577-8679	701 amino acids from the middle of the ENV protein, residues 106-806
SU del	1509 bases, nucleotides 6577-8085	503 amino acids of the SU protein, residues 106-608
V3/4 del	432 bases, nucleotides 7339-7770	144 amino acids of the V3 and V4 regions of SU, residues 360-503
V7/8 del	216 bases, nucleotides 8380-8595	72 amino acids from the V7 and V8 variable regions of the TM protein, residues 98-169
TM del	75 bases, nucleotides 8071-8145	25 amino acids in the cleavage junction between SU and TM
CT del	138 bases, nucleotides 8686-8823	46 amino acids from the cytoplasmic domain of TM
DU del	345 bases, nucleotides 4019-4363	115 amino acids from the DU protein, residues 9-123
IN del	669 bases, nucleotides 4418-5086	223 amino acids of the IN protein, residues 9-231
Vifn del	150 bases, nucleotides 5286-5435	50 amino acids from the N-terminal portion of the Vif protein, residues 19-68
Vifc del	438 bases, nucleotides 5436-5873	146 amino acids from the C-terminal portion of Vif, residues 69-214
Vif del	588 bases, nucleotides 5286-5873	196 amino acids from the Vif protein, residues 19-214
Orf(2) del	237 bases, nucleotides 5988-6224	79 amino acids of the ORF(2) protein
RRE del	84 bases, nucleotides 8827-8910	-

[0041] Examples of several specific mutations that produce attenuated viruses suitable for administration to animals to induce antibody production or for use in vaccines are MA del, ENV del, V3/4 del, V7/8 del, TM del, CT del, Vif del, Vifc del, Vifn del, ORF(2) del, CA del, NC del, SU del, IN del, DU del, and RRE del. Thus, viruses mutated in any of the Vif, MA, ORF(2), ENV, Vifn, Vifc, V3/4, V7/8, TM, CT, SU, CA, NC, IN, DU, or RRE genes are attenuated, and other nucleotide deletions or alterations that inactivate these genes should produce viruses with similar characteristics.

D. Generation of Antibodies to FIV-141 and Treatment of Infected Cats

[0042] Antibodies to FIV-141 can be produced in any of the animals typically used for antibody production, including mice, rabbits, etc. However, it is preferred that the antibodies be produced in cats. If wild type virus is used as antigen, the virus should be inactivated prior to administration. When attenuated viruses are used, e.g., viruses mutated so as to reduce or eliminate their infectivity, inactivation or the fixing of host cells is not required, although these procedures may be performed if desired.

[0043] Compositions containing virus may be administered to animals by any route, but animals will typically be injected intramuscularly, subcutaneously or intravenously. Generally, the virus preparation will include an adjuvant, e.

product. This can usually be most easily accomplished by deleting either the entire gene or a substantial portion of the gene. Although the specific methodology used for inducing mutations will vary depending upon the virus, the basic techniques are routine in the art and, using the procedures disclosed herein as a guide, can be readily carried out by a skilled molecular biologist. Antibody production, the making and administration of vaccines, *etc.*, may be accomplished as discussed in connection with FIV-141 herein, making minor adaptations as needed.

[0053] In addition, it is contemplated that antibodies to certain lentiviruses may be used to provide passive immunity to an animal or human infected with the virus. Either purified antibody or antibody-containing serum may be used for this purpose, and preparations may be administered on a periodic basis until an improvement in one or more signs associated with viral infection is observed.

EXAMPLES

Example 1: Construction of an Infectious FIV Proviral DNA Clone

A. Isolation and Cloning of FIV-141

Virus Isolation

[0054] FIV-141 was isolated from the plasma of an FIV-infected cat. The virus was amplified by administering plasma from the infected animal to a specific pathogen-free (SPF) cat. Infection of the inoculated cat was confirmed by virus isolation and seroconversion. The cat was euthanized 12 weeks post challenge, tissues were collected, and the spleen was used as the source of virus for the molecular cloning of the FIV-141 genome. Genomic DNA was isolated from the infected spleen using a DNA extraction kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. Purified genomic DNA was dissolved in TE buffer at a concentration of 1 ug/ml and stored at -70°C.

PCR Amplification and Cloning of Three Segments of the FIV-141 Genome

[0055] Three sets of oligonucleotides were designed based upon the published sequences of other FIV isolates (Talbot *et al.*, 1989, *Proc. Nat'l Acad. Sci. USA*, 86:5743-5747; Miyazawa *et al.*, 1991, *J. Virol.* 65:1572-1577; Talbot *et al.*, 1990, *J. Virol.* 64:4605-4613). These oligonucleotides were used to amplify three segments of the FIV-141 genome, one at the 5' end, one at the 3' end and one in the middle of the genome. Because of a low copy number of the FIV proviral genome in infected tissue, two rounds of PCR amplification were performed using a semi-nested set of primers for each segment.

[0056] Three primers were used to clone a segment from the 5' end of the FIV-141 proviral genome, extending from nucleotides 118 to 646. This region covers most of the 5' long terminal repeat, the intervening sequence between the 5' long terminal repeat and the Gag open reading frame, and the N-terminal portion of the Gag gene. The sequences of the primers were as follows: the forward primer pr-1 (117-CCGCAAAACACAT CCTATGTAAAGCTTGC-146, SEQ ID NO:2) and the two reverse primers, pr-2 (646-CGCCCCTGTCCATTCCCCATGTTGCTGTAG-617, SEQ ID NO:3) and pr-8 (1047-TTACTGTTTGAATAGGATATGCCTGTGGAG-1018, SEQ ID NO:4). First round PCR amplification was performed using 200 ng each of pr-1 and pr-8 as primers and 1 ug of genomic DNA as template, with a mixture of 0.5 units of *Taq* DNA polymerase (Gibco, BRL Gaithersburg, MD) and 1 unit of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Amplification proceeded at 94°C for one minute; followed by 30 cycles of denaturing at 94°C, for 45 seconds; annealing at 52°C for 45 seconds; and extension at 72°C for two minutes. The second round amplification was performed using primers pr-1 and pr-2 together with 2 ul of the first round PCR products as template. The same conditions used in the first round of amplification were applied in the second round, except that annealing took place at a temperature of 55°C.

[0057] Three oligonucleotides were also used to clone a segment from the 3' end of the FIV-141 proviral genome. This segment includes nucleotides 8874 to 9367, consisting of most of the 3' long terminal repeat and the intervening sequence between the 3' long terminal repeat and the ENV gene. The sequences of the three primers were as follows: the two forward primers, pr-5 (8793-GCAATGTGGCATGTCTGAAAAAGAGGAGGA-8822, SEQ ID NO:5) and pr-7 (8874-TCTTCCCTTTGAGGAAGATATGTCATATGAATCC-8907, SEQ ID NO:6), and the reverse primer, pr-6 (9367-TCTGTGGGAGCCTCAAGGGAGAACTC-9342, SEQ ID NO:7). Primers pr-5 and pr-6 were used to perform the first round amplification, and pr-6 and pr-7 were used to carry out the second round of amplification. The same conditions were applied to the present amplification as those used in the amplification of the segment from the 5' end of FIV-141 described above.

[0058] In order to clone a segment from the middle part of the FIV-141 genome, extending from nucleotides 5147 to 5631 and covering the C-terminal portion of the IN gene and the N-terminal portion of the Vif gene, a first round amplification was performed using the forward primer, pr-3 (4738-ACAAACAGATAATGGACCAAATTTTAAAAA-4767,

B. Molecular Characterization of the Cloned FIV-141 Virus**Sequence Results and Analysis of the Entire FIV-141 Genome**

5 [0065] The entire proviral genome of FIV-141 was found to contain 9464 bases. The genome is organized in a manner typical of lentiviruses and consists of: 5' and 3' long terminal repeats; three large open reading frames (ORF) containing the Gag, Pol, and ENV genes; and three small open reading frames containing the Vif, Rev, and ORF(2) regulatory proteins. The long terminal repeat shares 78.6% and 93.9% sequence homology with FIV-Petaluma (Olmsted *et al.*, 1989, Proc. Nat'l Acad. Sci. USA 86:2448-2452) and FIV-USIL (Sodora *et al.*, 1995, AIDS Res. Hum. Retroviruses 11: 10 531-533) isolates, respectively. The Gag polyprotein shares 88.4% and 94.4% amino acid homology with FIV-Petaluma and FIV-USIL isolates, respectively.

[0066] The Gag gene encodes the matrix (MA) protein (bases 627 to 1031), capsid (CA) protein (bases 1032 to 1724), and nucleocapsid (NC) protein (bases 1725 to 1976). The Gag and Pol polyprotein overlap 97 bases with the Pol ORF, beginning at nucleotide 1880 and ending at nucleotide 5239. A heptanucleotide frameshift signal (5'-GGGAAAC-3') is located 100 bases upstream of the 3' end of the overlap. As the result of a -1 frameshift during translation, a Gag/Pol polyprotein fusion is produced.

[0067] Compared with FIV-Petaluma and FIV-USIL isolates, the Pol polyprotein of FIV-141 exhibits an 85.7% and 92.2% amino acid identity, respectively. The Pol gene encodes: a lead sequence from nucleotide 1880 to 1978; a protease (PR) from nucleotide 1979 to 2326; a reverse transcriptase (RT) from nucleotide 2327 to 3994; a deoxyuridine triphosphatase (DU) from nucleotide 3995 to 4393; and an integrase (IN) from nucleotide 4394 to 5239.

[0068] The Vif ORF overlaps eight bases with the Pol gene, and shares 80.2% and 91.3% amino acid homology with FIV-Petaluma and FIV-USIL isolates, respectively. Immediately following the Vif gene is the ORF(2) gene, beginning at nucleotide 5988 and ending at 6224, which evidences a 62% and 92.4% sequence homology with FIV-Petaluma and FIV-USIL isolates, respectively.

25 [0069] The ENV polyprotein shares a 79.3% and 88.6% amino acid identity with FIV-Petaluma and FIV-USIL isolates, respectively. The ENV gene encodes: a surface (SU) protein, from nucleotide 6262 to 8088; and a transmembrane (TM) protein, from nucleotide 8089 to 8826.

[0070] The Rev protein results from the translation of a multiple splicing mRNA. The first exon of the putative Rev gene apparently shares an initiation codon with the ENV gene, beginning at nucleotide 6262 and ending at 6505. The second Rev exon begins at nucleotide 8947, extends into the U3 region of the 3' long terminal repeat, and ends at nucleotide 9161. The Rev protein of FIV-141 has a 67.3% and 83.9% amino acid homology with FIV-Petaluma and FIV-USIL isolates, respectively. The 151 base Rev responsible element (RRE) overlaps 52 bases with the ENV gene, beginning at nucleotide 8775 and ending at 8925.

35 [0071] Based on the sequence comparisons within the V3 region of the SU glycoprotein, FIV-141 is a type B isolate. Apparently, FIV-141 is most closely related to FIV-USIL, another type B FIV isolate.

C. Construction of a Full-Length Molecular Clone of FIV-141

40 [0072] In order to construct a full-length FIV-141 clone, the 20 bases at the extreme 3' end of the genome had to be added to the 3' half clone. In addition, a consensus sequence was identified by comparing the sequences from three independent clones. Site directed mutagenesis (SDM) was then used to adjust the sequences of the 5' and 3' half clones to match that of the consensus before construction of the full length viral clone.

Addition of Missing 20 Bases at the Extreme 3' End of FIV-141

45 [0073] In order to add the 20 bases to the 3' half clone of FIV-141, the long terminal repeat was first PCR amplified and cloned into a PCR-Script Amp SK(+) cloning vector using the 5' half clone as template and forward primer, PR-21 (5'-TTACAAGAATTCAACTGCAG TGGGAA GATTATTGGGATCCTGAAGAAAT-3', SEQ ID NO: 16) and reverse primer, pr-20 (5'-TTCAAGGAGCTCTTTTTCGACAACTGCGAGGTCCTGGCCC-3', SEQ ID NO: 17). In order to facilitate cloning the PCR fragment, two restriction enzyme sites (underlined), EcoRI and Pst I, were incorporated into the forward primer, PR-21, and two sites (underlined), Sac I and Sal I, were incorporated into the reverse primer, PR-20. The FIV-141 specific sequences in the primers are shown in italics. The resulting clone was sequenced and designated as pCR-LTR. A restriction fragment of pFIV-LTR generated by digestion with Sac I and Nhe I was cloned into one of the 3' half clones of FIV-141. The resulting clone was named pFIV3'-2A-1+ and the presence of the 20 bases at the extreme 55 3' end of FIV-141 was confirmed by nucleotide sequencing.

Oligo pF-7, designed to repair the error A5007T: 5'-GGCTCCTTATGAATTA
TACATACAACAGGAATCATTAGAATACAAGAC-3' (SEQ ID NO:24).

[0075] In order to make sequence changes in the 3' half of the genome, the 3' half clone "pFIV3'-2A-1+" was used as a template in performing SDM. There were nine changes in the pFIV3'-2A-1+ clone compared to the consensus sequence. Two nucleotide changes in the coding region were silent. The other seven changes all resulted in an amino acid substitution: one in the Vif protein (T5508C, H to Y); one in the ORF(2) region (A6041T, D to E); three in the SU protein (A6922G, V to I; G7007T, T to R; and A7814G, S to N); one in the TM region (A8405T, I to N); and one in the Rev region (A8976G, E to K). Seven mutagenesis oligonucleotides were designed for repairing these seven amino acid substitutions:

Oligo pF-8, designed to repair the error T5508C: 5'-CAAAATAGTTTAAGAT
TGTATGTTTATATAAGCAAT-3' (SEQ ID NO:25);

Oligo pF-9, designed to repair the error A6041T: 5'-CAGAAAAGTTAGATAGA
GAAGCAGCTATTAGATTGTTTAT-3' (SEQ ID NO:26);

Oligo pF-10, designed to repair the error A6922G: 5'-TAAAAGCAAATGTTA
ATATAAGTATACAAGAAGGACCTAC-3' (SEQ ID NO:27);

Oligo pF-11, designed to repair the error G7007T:
5'- AAAAGCTACAAGGCAATGCAGAAGGGGAAGGATATGGAAG-3'
(SEQ ID NO:28);

Oligo pF-12, designed to repair the error A7814G: 5'- AGAGGACCTTATTGT
ACAATTTAATATGACAAAAGCAGTGGAAA-3' (SEQ ID NO:29);

Oligo pF-13, designed to repair the error A8405T: 5'- CCCTCAATCTGTGG
ACAATGTATAACATGACTATAAATCA- 3' (SEQ ID NO:30);

assayed for FIV production, reverse transcriptase (RT) activity and viral infectivity.

FIV Production from Transfected CRFK Cells

- 5 [0083] Supernatants from the transfected CRFK cells were harvested on a daily basis after the transfection, and were assayed for FIV capsid protein production using the FIV Antigen Test Kit (IDEXX, Portland, ME), according to the protocol recommended by the manufacturer. This enzyme immunoassay was designed to detect the predominant group-associated antigen of FIV p26 capsid protein. FIV antigen p26 was detected at 24 hours post-transfection (PT), reached a peak at 72 hours PT, and then decreased to background levels at 11 days PT (see Figure 1).
- 10 [0084] In order to confirm virus production from the transfected (CRFK) cells, a reverse transcriptase (RT) activity assay (Boehringer Mannheim, Indianapolis, IN) was performed to detect virion-associated RT activity in the transfected cell supernatants. Briefly, 200 μ l of cell supernatant was harvested and spun 5 minutes in a microfuge to pellet cells and cell debris. Supernatants were centrifuged at 20,000 g for 20 minutes at 4°C in a swinging bucket rotor to pellet FIV virus particles. Viral particle pellets were resuspended in 40 μ l of lysis buffer from the kit, and assays were then
- 15 performed as recommended by the manufacturer. Virus production from transfected cells was demonstrated in cell supernatants 48 hours post-transfection (PT).

Infection of CRFK Cells

- 20 [0085] FIV-141 wild type virus does not infect CRFK cells. In order to determine whether the molecular clone virus exhibits similar behavior, CRFK cells were grown in 6 well plates and inoculated with 200 μ l of p26+ conditioned medium from transfected CRFK cells. After incubation for 2 hours at 37°C, cells were washed once with PBS, and 2 ml of RPMI 1640 medium supplemented with 3% FS was added to each well. Supernatants were then monitored for virus production by FIV p26 ELISA every 3 to 4 days post-transfection. It was found that, similar to the wild type virus, the FIV-141 clone
- 25 does not infect CRFK cells.

Infection of FeP2 Cells by Co-Culture with Transfected CRFK Cells

- [0086] CRFK cells were grown in 6 well plates and transfected as described above. At 48 hours post-transfection, 2 x 10⁶ FeP2 cells were added to each p26+ transfected well. After co-culture of cells for 72 hours, FeP2 cells (non-adherent) were separated from CRFK cells (adherent). The supernatants from the FeP2 cells were harvested and monitored for virus production by FIV p26 ELISA every 3 to 4 days. Four days post co-cultivation, high levels of virus production were demonstrated in the FeP2 cell supernatants (see Figure 2). Virus titer reached a plateau 6 days post-transfection, indicating that the FIV-141 molecular clone virus is infectious in FeP2 cells. The results also suggested
- 35 that infection of CRFK cells is blocked in an early stage of virus infection, *i.e.*, at the time of the entry of virus into cells.
- [0087] Overall, it was concluded that, upon transfection into CRFK cells, the FIV-141 molecular clone can replicate in the cell, and virus particles released from the cells are infectious for FeP2 T lymphocytes.

Infection of FeP2 Cells by Adsorption

- 40 [0088] FeP2 cells (2 x 10⁶) were suspended in 200 μ l of p26+ conditioned medium obtained from transfected CRFK cells and incubated at 37°C for two hours. Cells were washed with PBS, suspended in 2 ml Opti-MEM medium supplemented with 10% heat inactivated FCS, and incubated at 37°C. Supernatants were harvested and monitored for virus production by FIV p26 ELISA every three to four days. Four days post-infection, virus release from infected FeP2
- 45 cells was detected in the supernatants and reached a peak by three weeks post-infection (Figure 3). The results indicate that productive infection of FeP2 cells by FIV-141 can be achieved through either adsorption or co-culture with transfected CRFK cells. Compared to infection by co-cultivation, virus production reached a plateau much more slowly when infection took place by adsorption (Figures 2 and 3).

50 Example 3: Mutant FIV-141 Clones and Their Use in Vaccines

[0089] In order to develop FIV-141 vaccine candidates, the infectious FIV-141 wild-type clone was used to construct a number of gene-deleted clones. The general criteria for making the mutant clones are:

- 55 1. The deletions or mutations introduced into the FIV-141 genome must be severe enough so that virus infectivity is substantially reduced (attenuated) after the clones are transfected into cultured cells *in vitro* or administered to cats *in vivo*.
2. The deletions or mutations introduced into the FIV-141 genome should not abolish the replication competency

nucleotides 6577 to 8679, corresponding to the middle 701 amino acids of the ENV protein (residues 106 to 806). The N-terminal 105 residues, the majority of which overlaps the first exon of the Rev protein, and the C-terminal 45 residues which overlaps with the Rev responsible element (RRE), were maintained in the deletion clone.

5 SU del Mutation

[0098] Two Spe I sites were generated in the SU region of FIV-141 by SDM using clone pFIV3'-2A-1+/M-21 as template and, as mutagenesis primers, Mpsu-1 (5'-GAGGTATAAAGG TAAACAAAAAACTAGTGCCATTCATAT-TATGTTAGCCCTTGC-3', SEQ ID NO:38) and Mpsu-2 (5'-ACTAACTATAGTCTATTTACTAACAACTAGTTTGAGA-TATTAATAAGCCAT AGAAAC-3', SEQ ID NO:39). The Spe I fragment was deleted by Spe I digestion followed by self ligation of the large remaining fragment. The resulting clone was ligated to pFIV5'-D-11/M-52. This clone has a deletion of 1509 basis from nucleotides 6577 to 8085, corresponding to a deletion of 503 amino acids (residues 106 to 608) of the SU protein. The clone maintains the N-terminal 105 amino acids of the SU protein.

15 V3/4 del Mutation

[0099] SDM was performed to create two Sph I sites flanking the V3 and V4 region of the SU protein. The clone pFIV3'-2A-1+/M-21 was used as template along with the mutagenesis primers: Mpenv-5 (5'-ATACCGAAATGTGGAT-GGTGGAATCAGGCATGCTATTATAAT AATTGTAAATGGGAAGAAGC-3', SEQ ID NO:40) and Mpenv-6 (5'-GCAC-TATGTACAATTG TTCCTTACAGGCATGCTTCACTATGAAAATAGAGGACCTTAT-3', SEQ ID NO:41). Sph I sites are underlined. After digestion to remove the Sph I fragment, the clone was self-ligated and then joined to pFIV5'-D-11/M-52. This clone contains a deletion of 432 bases from position 7339 to 7770, corresponding to a deletion of 144 amino acids (from residue 360 to 503) of the SU protein, covering the V3 and V4 regions.

25 V7/8 del Mutation

[0100] SDM was used to create two Sph I sites flanking the V7 and V8 region of the TM protein. This was accomplished using the clone pFIV3'-2A-1+/M-21 as template and the mutagenesis primers Mpenv-7 (5'-GAATCAATTCCTTTT-GTAAGATCGCATGC AATCTGTGGACAATGTATAACATGACTA-3', SEQ ID NO:42) and Mpenv-8 (5'-GGGAAAATT-GGGTGGGATGGATAGGTAAGATCGCATGCTATTTAAAGGACTTCTTGGT AG-3', SEQ ID NO:43). Sph I sites are underlined. Digestion with Sph I resulted in the elimination of 216 bases from nucleotides 8380 to 8595, and was followed by ligation of the large fragment. The resulting clone was then joined to the 5' half clone pFIV5'-D-11/M-52 to generate "V7/8 del." This contains the deletion of 72 amino acids (from residues 98 to 169) of the TM protein covering the V7 and V8 various regions.

35 TM del Mutation

[0101] The 3' half clone of FIV-141 has a unique Age I site at nucleotide 8145. SDM was performed to create a second Age I site at position 8071. The 3' half clone was used as template along with the mutagenesis primer, Mpenv-3 (5'-GGAAGAAGTTATGAG GTATACCGGT AAACAAAAAAGGGCC-3', SEQ ID NO:44). A 75-base fragment between the two Age I sites was deleted by restriction enzyme digestion followed by self-ligation of the large restriction fragment. The resulting clone was ligated to the 5' half clone to generate "TM del." This contains a deletion of 25 amino acids in the cleavage junction between the SU and TM proteins. The deleted amino acids include 6 C-terminal residues of the SU protein (4 of which are basic (either K or R) and required for the processing of the SU/TM cleavage site) and 19 N-terminal residues of the fusion peptide of the TM protein (required for membrane fusion between virion envelope and cell membrane).

45 CT del Mutation

[0102] SDM was performed to truncate the cytoplasmic tail of the TM protein using the 3' half clone pFIV3'-2A-1+/M-21 as template and the mutagenesis primer, Mpenv-4 (5'-CTACTTATATGCTTGCCCTACATTGGTC-GACTGATAGTGAACTGTACTAATAAAATATTGG G-3', SEQ ID NO:45). A Sal I restriction site (underlined) was incorporated into the oligonucleotides by silent mutation to facilitate the screening of mutants. Three tandemly repeated translation stop codons (italicized) were incorporated in the primer right after the transmembrane domain of the TM protein. The resulting clone was ligated to the 5' half clone to generate "CT del." This has a 138-base truncation from nucleotides 8686 to 8823 (corresponding to a truncation of 46 amino acids from the cytoplasmic domain of the TM protein).

was accomplished using pFIV3'-2A-1*/M-21 as template and, as mutagenesis primers, Mporf-1 (5'-GTGGACGGGA-GAATTATGAACGCGTGAACCTAATC CCACTGTTTAATAAGGTTACAG-3', SEQ ID NO:52) and Mporf-2 (5'-CTACAT-TATC CATAAATACTGCCTAGACGCGTTTCTTTTAATATTCATCTGCAG-3', SEQ ID NO: 53). Mlu I sites in the primers are underlined. In addition to the two Mlu I sites created by SDM, there is an Mlu I site at nucleotide 5436 in the clone. To construct "ORF(2) del," a 5.4 kb Mlu I/Xho I fragment from the 5' half clone pFIV5'-D-11/M-52 was ligated to the large Mlu I/Xho I fragment of the 3' half clone pFIV3'-2A-1*/M-21. A 552 base Mlu I fragment from position 5436 to 5988 was then inserted into the resulting clone. ORF(2) del contains a deletion of 237 bases, covering the entire ORF (2) gene.

10 RRE del Mutation

[0111] SDM was performed to create two Spe I sites in the RRE region using pFIV3'-2A-1*/M-21 as template and the mutagenesis primers, Mprre-1 (5'-GGCATATCTGAA AAAGAGGAGGAATGAACCTAGTATATCAGACCTGTAGAA-TACA-3', SEQ ID NO:54) and Mprre-2 (5'-GAGGAGGATGTGTCATATGAATCAAATACTAGTCAAAAATAACAGT-AAAATCT ATATTG-3', SEQ ID NO:55). Spe I sites in the primers are underlined. Deletion of the Spe I fragment was achieved by Spe I digestion followed by self ligation of the large fragment. The resulting deletion clone was ligated to pFIV5'-D-11/M-52 to generate "RRE del." This contains a deletion of 84 bases from nucleotide 8827 to 8910.

20 Example 4 Characterization of the FIV-141 Gene Deletion Clones

A. Viral Protein Expression and/or Defective Virus Production

[0112] Each plasmid of the deletion clones was transfected into CRFK cells as previously described. FIV p26 ELISA assays were performed to detect viral protein expression and/or virus particle production in the transfected cell supernatants. At 48 hours post-transfection, samples from 13 of the constructs were found to produce a strong positive signal comparable to that observed for the wild type FIV-141 molecular clone (see Figure 4). The highest levels of virus particle production were observed for the six deletion clones in the ENV region, including ENV del, TMf del, SU del, CT del, V3/4 del and V7/8 del.

[0113] Comparable levels of virus particle production were obtained for seven other deletion clones, including three deletion clones in the Vif region (Vifn del, Vifc del and Vif del), MA del, DU del, IN del and ORF(2) del. The results indicate that the deletions carried by these 13 clones do not interfere with the formation and release of virus particles from the transfected cells. A relatively weak positive signal was detected for NC del, indicating that deletion in this region affects virus particle assembly or release.

[0114] No virus particle production was detected in the supernatants of cells transfected with CA del or with RRE del. The deletion in the C-terminus of the CA protein may either abolish virus particle formation or result in loss of the epitope recognized by the monoclonal antibody (MAb) used in the p26 ELISA kit. As expected, deletion in the RRE region resulted in a block of the export of unspliced viral RNA from the nucleus to the cytoplasm, leading to either a total lack of, or a dramatic decrease in, the expression of viral structural proteins.

40 B. Intracellular RT-PCR to Detect Viral RNA Expression

[0115] Intracellular RT-PCR was performed to detect viral RNA expression in the two deletion clones, CA del and RRE del. Plasmid DNA for each clone was transfected into different CRFK cells. Forty-eight hours after transfection, total RNA was isolated from the transfected cells using an RNeasy kit (Qiagen, Chatsworth, CA). The RNA was eluted in 50 ul of DEPC water, and 2 ul of each RNA sample was used to synthesize the first strand of cDNA using Superscript II (Gibco BRL, Gaithersburg, MD).

[0116] A 585 base pair fragment from nucleotides 2958 to 3542 was amplified using as a forward primer Sp-8 (5'-TATTATGGTGGGGATTGAAAC-3', SEQ ID NO:56) and, as a reverse primer, Sp-20 (5'-TAATTAGATTTGATTCAG-GC-3', SEQ ID NO:57). Two ul of cDNA from each reaction and, as a control, 2 ul of total RNA from each preparation, were used as the template in PCR reactions. Each reaction was performed in a volume of 100 ul using a PCR amplification kit (Gibco BRL, Gaithersburg). The reaction proceeded as follows: 25 cycles at 94°C for 30 seconds; 55°C for 30 seconds; and 72°C for another 30 seconds. Ten ul from each reaction was loaded on a 1% agarose gel. A specific band with the expected size was observed for both CA del and RRE del clones, indicating that viral RNA expression occurred in the cells transfected with these clones. The results suggest that the failure to detect p26 protein expression by ELISA for CA del is probably due to either a failure of virus particle formation or a lack of the epitope recognized by the antibody used in the p26 ELISA assay. For the RRE deletion clone, viral gene expression was demonstrated by intracellular RT-PCR, but no p26 protein expression was detected using the ELISA assay. The discrepancy may reflect a much higher sensitivity of RT-PCR assay when compared to the ELISA.

However, the expressed recombinant protein maintains its antigenic properties, as evidenced by its interaction with monoclonal antibodies as determined using Western Blots and radioimmunoprecipitation assays (Rimmelzwaan *et al.*, 1994, J. Gen. Virol. 75:2097-2012). Upon transfection into CRFK cells, the deletion clone produces defective virus particles at a level comparable to a wild type FIV-141 clone. The defective viral genome and RT enzymes were encapsidated in the defective virions.

SU del

[0124] SU del had a deletion of 503 amino acids from residue 106 to 608 of the SU protein. It was found to maintain levels of virus particle production approximately equal to that of the wild type clone. Both the gene-deleted viral genome and RT enzyme were encapsidated. However, in contrast to ENV del, cells transfected by SU del produced virus particles that are infectious in the FeP2 cells, although to a much lesser extent than the wild type virus. Thus, it appears that deletion of the SU protein from the FIV-141 genome attenuated the virus. It is believed that FIV binding to cellular receptors, which is the first step in virus infection, is mediated by the SU protein when associated with the TM protein. The mechanism by which the mutant virus binds to and enters target cells is unknown. An alternative pathway for the mutant virus to enter host cells may be responsible for the observed lower infectivity associated with the deletion clone.

V3/4 del and V7/8 del

[0125] One hundred forty-four amino acids from residues 360 to 503 of the SU protein (covering the V3 and V4 variable regions), and 72 amino acids from residues 98 to 169 of the TM protein (encompassing the V7 and V8 regions) were deleted in V3/4 del and V7/8 del respectively. Upon transfection into CRFK cells, each clone produced defective virus at levels similar to that observed for the wild type clone. As with other ENV-related deletion clones, V3/4 del and V7/8 del encapsidated their gene-deleted viral genomes and RT enzymes into virions. The infectivity assay indicated that deletion of the V3 and V4 region of SU, and deletion of the V7 and V8 region in the TM protein, totally abolished virus infectivity in the FeP2 cells. The V3 variable region is the immunodominant domain and has been reported to be involved in multiple functions, including virus tropism, viral pathogenesis, and neutralizing epitopes. It is not presently clear at which step viral infection was blocked in these two deletion clones.

CT del

[0126] The TM protein of FIV has a relatively long cytoplasmic tail (46 amino acids in length). Truncation of this tail in CT del clone resulted in a loss of infectivity of the virus in FeP2 cells. However, truncation had no effect on virus particle formation and encapsidation of the viral genome and RT protein. A specific functional interaction between MA and the TM cytoplasmic tail has been reported for FIV as well as for HIV-1. This interaction has been proposed to be important for the incorporation of the ENV protein into virions. Truncation of the cytoplasmic domain in CT del may eliminate the functional interaction between the MA and TM proteins, thereby blocking the incorporation of ENV.

MA del

[0127] MA del contains a 41 amino acid deletion from residues 85 to 125 at the C-terminus of the MA protein. Upon transfection into CRFK cells, the clone produced defective virus at a level comparable to that produced using the wild type FIV-141 clone. This indicates that deletion at the C-terminus domain has no significant effect on virus particle assembly and release. The gene-deleted viral genome and RT protein were encapsidated in the defective virus particles. When these virus particles were released from transfected CRFK cells, they were non-infectious with respect to FeP2 cells.

CA del

[0128] A deletion of 38 amino acids from residues 9 to 46 at the N-terminus of CA protein abolished viral particle formation, as evidenced by a negative signal in the p26 ELISA assay, the intra-virion RT PCR assay, and the RT activity assay. However, intracellular RT PCR from the transfected CRFK cells demonstrated that the deletion did not block viral RNA expression. Therefore, the failure to detect p26 protein or defective virus production in the supernatants of transfected cells is due to the block in viral particle assembly, not in viral protein expression.

NC del

[0129] The entire NC protein was deleted in the NC del clone. Cells transfected with this clone produced defective

EP 0 997 529 A2

	ATCC Accession No.
Viral strain FIV-141	VR-2619
Plasmid pFIV-141-B1	203001

5

[0136] All patents, patent applications, and publications cited above are incorporated herein by reference in their entirety.

10 [0137] The present invention is not to be limited in scope by the specific embodiments described, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent compositions and methods are within the scope of the invention.

15

20

25

30

35

40

45

50

55

EP 0 997 529 A2

	ccaaatctcc	ccgagcagta	caattgaagc	agggagctaa	agaggactat	tcctcattca	1500
	tagatagact	atttgctcaa	atagatcaag	agcagaacac	agctgaggta	aagctgtatt	1560
	taaaacaatc	tttgagcata	gcaaatgcta	atccagattg	taagagagcg	atgagtcac	1620
5	ttaaaccaga	aagtacttta	gaagagaaac	tgagagcctg	ccaggaaata	ggatcgccag	1680
	gatacaaaat	gcaactattg	gcagaggctc	ttactagggt	gcaaacagtt	caagcaaaaag	1740
	gaccaaggcc	agtatgtttc	aattgtaaaa	aaccaggaca	cctggccaga	caatgtagac	1800
	aagcaaagag	atgtaataaa	tgtggaaaac	ctggtcactt	agctgctaac	tgttggcaag	1860
	gaggtaaaaa	gtccccggga	aacggggcga	tggggcgagc	tgacgcccc	gtaaatcaag	1920
10	tgacgcaagt	gataccatct	gcacccccgg	tagaggagaa	attgttagat	atgtaaacta	1980
	taataaagtg	ggtaccacca	caactttaga	aaaaagacct	gaaatacaaa	tattcgtaaa	2040
	tgggtatcct	ataaaatttt	tattagatac	aggagcagat	ataacaattt	taaacagaaa	2100
	agactttcag	atagggaatt	ctatagaaaa	tgggaaacag	aatatgattg	gagtaggagg	2160
15	cggaaagaga	ggaacaaatt	atatcaatgt	gcatttagaa	attagagatg	aaaattataa	2220
	gacacagtgt	atatttgga	atgtgtgtgt	cttggaggat	aattcattaa	tacaaccatt	2280
	attgggaaga	gataacatga	ttaagttcaa	cataagggtg	gtaattggctc	aaatttcaga	2340
	gaaaattcca	atagtaaaag	taagaatgaa	agaccctact	caagggcctc	aggtaaaaca	2400
	atggccatta	tcaaatgaga	aaattgaagc	tctaactgac	atagtaaaaca	ggttagaaca	2460
20	agagggaaag	gtaaaaagag	ctgatccaaa	taatccttgg	aacactcccg	tatttgcaat	2520
	caagaaaaag	aatggtaaat	ggagaatgct	catagatttt	agggtcctaa	ataaattaac	2580
	agacaaaggg	gcagaagttc	agttaggact	ccctcatcct	gctggattac	aattgaaaaa	2640
	acaagtaact	gtattggaca	taggggacgc	atattttact	attcctctag	atccagatta	2700
25	tgctccttat	actgcattta	cactacctag	aaaaaacaat	gcaggaccag	ggaggagata	2760
	catatggtgt	agtttaccac	agggtgggt	cttgagtcca	ttgatataat	agaggacctt	2820
	agacaatata	ctccaacctt	ttattaaaca	gaatcctgag	ttagatattt	atcaatatat	2880
	ggatgatata	tatataggat	caaattttaag	taaaaaggaa	cataaactaa	aagtagaaga	2940
	attaagaaaa	ttgttattat	ggtggggatt	tgaaaccccg	gaagataaat	tacaagaaga	3000
30	gcccccttat	aagtggatgg	gctatgaatt	acatccatta	acgtggtcaa	tacagcaaaa	3060
	gcaattagaa	attccagaga	gaccacatt	aatgaatta	cagaagttag	caggtaagat	3120
	taactgggct	agtcaaacca	ttccagactt	gagcataaaa	gaactaacta	atatgatgag	3180
	aggagatcaa	aagttagact	caataagaga	atggacgaca	gaggccaaga	atgaagtgga	3240
35	gaaagctaa	agagcaattg	agacacaggc	acagctagga	tattatgatc	ctaatacgaga	3300
	attatatgct	aaattaagtc	ttgtgggacc	acatcaacta	agctatcagg	tgtatcataa	3360
	aaaccagaa	cagatattat	ggtatgggaa	aatgaatagg	cagaagaaaa	aagcagaaaa	3420
	tacttgtgat	atagctctaa	gggcatgtta	caaaataaga	gaagaatcca	ttataagaat	3480
	aggaaaaaga	ccagtatatg	aaatacctac	atccagagaa	gcttgggaat	caaatactaat	3540
40	tagatctcca	tatcttaagg	cctcaccacc	tgaggtggaa	tttatacatg	ctgccttaaa	3600
	tataaaaaaga	gctctaagca	tgatacaaga	tgccctata	ttgggagcag	aaacatggta	3660
	catagatggg	ggaagaaaac	aaggaaaagc	agcaagagca	gcttattgga	cagatacggg	3720
	cagatggcag	gtaatggaaa	tagaagggaag	taatcaaaaa	gcagaagtac	aagctttatt	3780
45	attggcccta	caggcaggac	cagaggaaat	gaatattata	acagattcac	aatatattgt	3840
	gaatattatt	aatcaacaac	cagatttgat	ggaagggaat	tggcaagaag	tcttagaaga	3900
	aatggaaaag	aaagtagcaa	tctttataga	ttgggtacct	ggacataaag	gtattccagg	3960
	aaataaagag	gtagatgaac	tttgtcaaac	gatgatggtt	atagaaggtg	aaggaatatt	4020
	agataaaaga	tcagaagatg	caggatatga	tttattagct	gcacaagaaa	tacatctctt	4080
50	gcctggggag	gtaagagtag	taccaacaag	aacaaagata	atgttaccta	aaggatattg	4140
	gggattaata	atgggaaaaa	gttcaatggg	aagcaaagga	ttagatgtat	taggaggagt	4200
	tatagatgaa	ggatatagag	gagaattagg	ggtgataatg	attaacctat	ctaaaaaatc	4260
	aataacatta	tcagaaaaac	aaaaagtagc	acaattaata	atattacctt	gtaaacatga	4320
55	aagcttacaa	caaggagaaa	taataatgga	ttcagaaaga	ggaagaaagg	gatttgggtc	4380
	aactggagtc	ttttcttcat	gggtggacag	aattgaggaa	gcagaattaa	atcatgaaaa	4440

EP 0 997 529 A2

5 tggagacaaa gaaacagatg ggagtggagg ccagactttg aaagtgagaa agtaaaaaata 7500
 tcattacaat gtaatagtag aaaaaattta acttttgcaa tgagaagtgc aagtgttat 7560
 tatgatgtac aaggagcatg gatagaattt ggatgttata gaaataaatc aagaacccat 7620
 10 acgggagcaa gatttagaat aagatgtaaa tggaatgaag gaaagaatct atctctcatt 7680
 gatacatgtg ggactacttc aaatgtgaca ggagccaacc ctgtagattg tactatgaaa 7740
 acaagcacta tgtacaattg ttccttacia gatagtttca ctatgaaaat agaggacctt 7800
 attgtacaat ttaatatgac aaaagcagtg gaaatgtata atattgctgg gaattgggtc 7860
 15 tgtacatctg atttaccac aggggtgggga tatatgaaat gtaattgtac aaatgccact 7920
 gatggggaga ataaaatgaa atgccctagg aatcagggtg ttttaagaaa ctggtacaat 7980
 ccagttgcag gactaagaca agctcttatg aagtatcaag tagtaaaaca accagaatat 8040
 ttgggtgtac cggaagaagt tatgaggtat aaaggtaaac aaaaaagggc cgctattcat 8100
 attatgttag cccttgctac ggtgttatct atagctggag caggaaccgg tgccactgct 8160
 20 attgggatgg tgacacacta tcagcaagtt ttggctacc atcagcaggc attggacaaa 8220
 ataactgagg cactgaaaat aaacaactta aggttaatca ctttagaaca tcaagtatta 8280
 gtgatagggt taaaagtaga ggctatagaa aaattcctat atacagcttt tgctatgcaa 8340
 gaattaggat gtaatcagaa tcaattcttt tgtaagattc ccctcaatct gtggacaatg 8400
 25 tataacatga ctataaatca tacactatgg aatcatggaa atataacttt gggagaatgg 8460
 tataatcaaa caaaaagttt acaagaaaaa ttttatgaga taattatgga tatagaacaa 8520
 aataatgtac aagggaaaaa tggaatacaa caattacaaa aatgggaaaa ttgggtggga 8580
 tggataggca aaatccctca atatttaaaa ggacttcttg gtagtgtgtt gggaatagga 8640
 ctaggaatct tactactact tatatgcttg cctacattag tagattgtat aagaaactgt 8700
 30 actaataaaa tattgggata tacagttatt gcaatgcctg aaatagatga tgaggaagta 8760
 caccatcag tggaattgag gagaaatggc aggcaatgtg gcatatctga aaaagaggag 8820
 gaatgatgga gcatttcaga cctgtagaat acaggagtaa tgctgagctg agttcttccc 8880
 tttgaggagg atgtgtcata tgaatccatt tcaaatcaaa aataacagta aaatctatat 8940
 35 tgtaaggcaa acgaaaaaga caacgcagaa gaagaaagaa gaaggccttc aaaaaattga 9000
 tgctggattt agaggctcga tttaaagcgt tgtttgaaac accttcagct acagaatata 9060
 ctgcagacga gacagaagaa gagactcttg aaaaagaaaa aagggtggac tgggaagatt 9120
 attgggatcc tgaagaaata gaaaaaatgc taatggactg aggacgtaca taaacaagt 9180
 acagatggaa acagctgaat atgactcaat gctagcagct gcttaaccgc aaaaccacat 9240
 40 cctatgtaaa gcttgccgat gacgtgtatc ttgctccatt ataagagtat ataaccagt 9300
 ttttgtaaaa gcttcgagga gtctctctgt tgagggcttt cgagttctcc cttgaggctc 9360
 ccacagatac aataaaaaac tgagctttga gattgaaccc tgtcttgtat ctgtgtaatt 9420
 tctcttacct gcgaatccct ggagtccggg ccaggacact cgca 9464
 45 <210> 2
 <211> 30
 <212> DNA
 <213> Feline immunodeficiency virus
 50 <400> 2
 ccgcaaaacc acatcctatg taaagcttgc 30
 <210> 3
 55 <211> 30
 <212> DNA
 <213> Feline immunodeficiency virus
 <400> 3
 60 cgccccgtgc cattccccat gttgctgtag 30

EP 0 997 529 A2

<211> 30
 <212> DNA
 <213> Feline immunodeficiency virus
 5
 <400> 10
 ttaaaggatg aagagaaggg atattttctt 30
 10
 <210> 11
 <211> 30
 <212> DNA
 <213> Feline immunodeficiency virus
 15
 <400> 11
 tgggaagatt attgggatcc tgaagaaata 30
 20
 <210> 12
 <211> 40
 <212> DNA
 <213> Feline immunodeficiency virus
 25
 <400> 12
 catatcctat ataataatca cgcgtatgaa agctccacct 40
 30
 <210> 13
 <211> 24
 <212> DNA
 <213> Feline immunodeficiency virus
 <400> 13
 tgcgaggtcc ctggcccga ctcc 24
 35
 <210> 14
 <211> 40
 <212> DNA
 <213> Feline immunodeficiency virus
 40
 <400> 14
 aggtggagct ttcatacgcg tgattattat ataggatatg 40
 45
 <210> 15
 <211> 29
 <212> DNA
 <213> Feline immunodeficiency virus
 50
 <400> 15
 ctccagggat tcggaggtaa gagaaatta 29
 55
 <210> 16
 <211> 49
 <212> DNA

EP 0 997 529 A2

	<400> 22	
	agaaagggat ttgggtcaac tggagtcttt tcttcatggg tgga	44
5	<210> 23	
	<211> 51	
	<212> DNA	
	<213> Feline immunodeficiency virus	
10	<400> 23	
	gggggacaat taaagattgg acctggcata tggcaaattgg actgtacaca c	51
	<210> 24	
15	<211> 49	
	<212> DNA	
	<213> Feline immunodeficiency virus	
20	<400> 24	
	ggctccttat gaattataca tacaacagga atcattaaga atacaagac	49
	<210> 25	
	<211> 36	
25	<212> DNA	
	<213> Feline immunodeficiency virus	
	<400> 25	
30	caaaatagtt taagattgta tgtttatata agcaat	36
	<210> 26	
	<211> 40	
	<212> DNA	
35	<213> Feline immunodeficiency virus	
	<400> 26	
	cagaaaagtt agatagagaa gcagctatta gattgtttat	40
40	<210> 27	
	<211> 40	
	<212> DNA	
	<213> Feline immunodeficiency virus	
45	<400> 27	
	taaaagcaaa tgtaatatata agtatacaag aaggacctac	40
	<210> 28	
50	<211> 40	
	<212> DNA	
	<213> Feline immunodeficiency virus	
55	<400> 28	
	aaaagctaca aggcaatgca gaaggggaag gatatggaag	40

EP 0 997 529 A2

<211> 51
 <212> DNA
 <213> Feline immunodeficiency virus
 5 <400> 35
 acagcctttt cagctaattt aactagtact gatatggcta cattaattat g 51
 <210> 36
 10 <211> 50
 <212> DNA
 <213> Feline immunodeficiency virus
 <400> 36
 15 actatagtct atttactaac tggttacctg agatatttaa taagccatag 50
 <210> 37
 <211> 54
 20 <212> DNA
 <213> Feline immunodeficiency virus
 <400> 37
 25 tacttatatg cttgcctaca ttgggttacc gtataagaaa ctgtactaat aaaa 54
 <210> 38
 <211> 54
 <212> DNA
 30 <213> Feline immunodeficiency virus
 <400> 38
 gaggtataaa ggtaaacaaa aaactagtgc cattcatatt atggttagccc ttgc 54
 35 <210> 39
 <211> 58
 <212> DNA
 <213> Feline immunodeficiency virus
 40 <400> 39
 actaactata gtctatttac taacaactag tttgagatat ttaataagcc atagaaac 58
 <210> 40
 45 <211> 62
 <212> DNA
 <213> Feline immunodeficiency virus
 <400> 40
 50 ataccgaaat gtggatggtg gaatcaggca tgctattata ataattgtaa atgggaagaa 60
 gc 62
 <210> 41
 55 <211> 58

EP 0 997 529 A2

<212> DNA
<213> Feline immunodeficiency virus

5 <400> 47
gaaataataa tggattcaga aagaggaact agtggatttg ggtcaactgg agtcttttc 59

<210> 48
<211> 57
10 <212> DNA
<213> Feline immunodeficiency virus

<400> 48
15 cttcatgggt ggacagaatt gaaactagtg tattaaatca tgaaaaattt cactcag 57

<210> 49
<211> 59
<212> DNA
20 <213> Feline immunodeficiency virus

<400> 49
gcaatgggtg tattataaag atcagactag taaaaagtgg aagggaccaa tgagagtag 59

25 <210> 50
<211> 57
<212> DNA
<213> Feline immunodeficiency virus

30 <400> 50
agaagactct ttgcagttct ccaatgaacg cgtagagtgc ccatgttata catatcg 57

35 <210> 51
<211> 44
<212> DNA
<213> Feline immunodeficiency virus

40 <400> 51
cgtgtggcaa agaggctaaa acgcgtagag gctgttgtaa tcag 44

<210> 52
<211> 56
45 <212> DNA
<213> Feline immunodeficiency virus

<400> 52
50 gtggacggga gaattatgaa cgcgtgaact aatcccactg ttaataagg ttacag 56

<210> 53
<211> 55
<212> DNA
55 <213> Feline immunodeficiency virus

8. FIV progeny virus produced by the host cell of claim 7.
9. A fixed cell vaccine comprising a host cell infected with the virus of claim 1, or transfected with the nucleic acid of claim 5, wherein said host cell has been fixed, and a pharmaceutically acceptable carrier.
10. An attenuated FIV-141 virus which replicates upon entry into a host cell but which exhibits significantly reduced infectivity to feline T lymphocytes when compared to the wild type FIV-141 virus, wherein said attenuated virus is produced by mutating one or more genes in the FIV-141 genome selected from the group consisting of: Vif, MA, ORF(2), ENV, CA, NC, SU, TMf, CT, IN, DU, V3/4, V7/8, and RRE.
11. The attenuated FIV-141 virus of claim 10, wherein said gene is selected from the group consisting of Vif, MA, ORF(2), and ENV.
12. A host cell infected with the attenuated virus of claim 10 or claim 11.
13. Attenuated FIV-141 virus produced in the host cell of claim 12.
14. An attenuated whole virus vaccine, comprising the virus of either claim 10 or claim 11, and a pharmaceutically acceptable carrier.
15. An attenuated host cell vaccine comprising the host cell of claim 12, and a pharmaceutically acceptable carrier.
16. A substantially purified FIV-141 nucleic acid molecule having a sequence corresponding to SEQ ID NO:1, but wherein said nucleic acid molecule is mutated in one or more genes selected from the group consisting of Vif, MA, CA, NC, SU, TMf, ORF(2), CT, ENV, Vifn, Vifc, IN, DU, V3/4, V7/8, and RRE, such that when the mutated nucleic acid molecule has been introduced into a host cell, the host cell produces an attenuated FIV-141 virus that replicates but has significantly reduced infectivity in peripheral blood mononuclear cells (PBMCs) relative to the wild type FIV-141 virus.
17. The nucleic acid molecule of claim 16, wherein said gene is selected from the group consisting of: MA, Vif, ORF(2), and ENV.
18. The nucleic acid molecule of claim 16 or 17, wherein said nucleic acid is DNA.
19. A host cell transfected with the nucleic acid molecule of claim 16, 17 or 18.
20. FIV progeny virus produced by the host cell of claim 19.
21. A vaccine comprising the nucleic acid molecule of claim 16, 17 or 18 at a concentration sufficient to induce immunity when administered to a cat, and a pharmaceutically acceptable carrier.
22. The vaccine of claim 21, wherein said nucleic acid is DNA.
23. A vaccine comprising a host cell transfected with the nucleic acid molecule of claim 16, 17 or 18, and a pharmaceutically acceptable carrier.
24. The vaccine of claim 23, wherein said host cell has been fixed.
25. A method of making an attenuated lentivirus that replicates in host cells but that has significantly reduced infectivity relative to its wild type counterpart, said method comprising mutating one or more genes of the lentivirus selected from the group consisting of: MA, CA, NC, DU, ENV, SU, TMf, CT, V3/4, V7/8, Vif, Vifn, Vifc, IN, RRE, and ORF(2).
26. The method of claim 25, wherein said one or more genes are selected from the group consisting of MA, ORF(2), and ENV.
27. The method of claim 25 or 26, wherein said lentivirus is a strain of FIV.
28. An attenuated lentivirus produced by the method of claim 25 or claim 26.

EP 0 997 529 A2

48. The method of claim 47, wherein said virus is inactivated prior to administration.
49. The method of claim 47, wherein said host cell is fixed prior to administration.
- 5 50. The method of claims 47, 48 or 49, further comprising purifying said antibodies from said mammal.
51. An antibody produced by the method of claim 47, 48, 49 or 50.
- 10 52. A method of inducing an immune response in a cat, comprising administering the vaccine of claim 4, 9, 14, 15, 21, 22, 23, 24, 30, 31, 37, 38 or 39 to said cat at a dosage sufficient to induce protective immunity against subsequent infection with FIV-141.
- 15 53. A method of treating a mammal for lentivirus infection, comprising administering the antibody of claim 51 to said mammal at a dosage sufficient to reduce one or more symptoms associated with said infection.
54. A method of inducing an immune response in a mammal, comprising administering the vaccine of claim 30, 31, 37, 38 or 39 to said mammal at a dosage sufficient to induce protective immunity against subsequent infection with at least one strain of said lentivirus.
- 20 55. The method of claim 54, wherein said mammal is a cat, said lentivirus is a strain of FIV and said vaccine is administered at a dosage sufficient to induce protective immunity against subsequent infection by at least one strain of FIV.

25

30

35

40

45

50

55

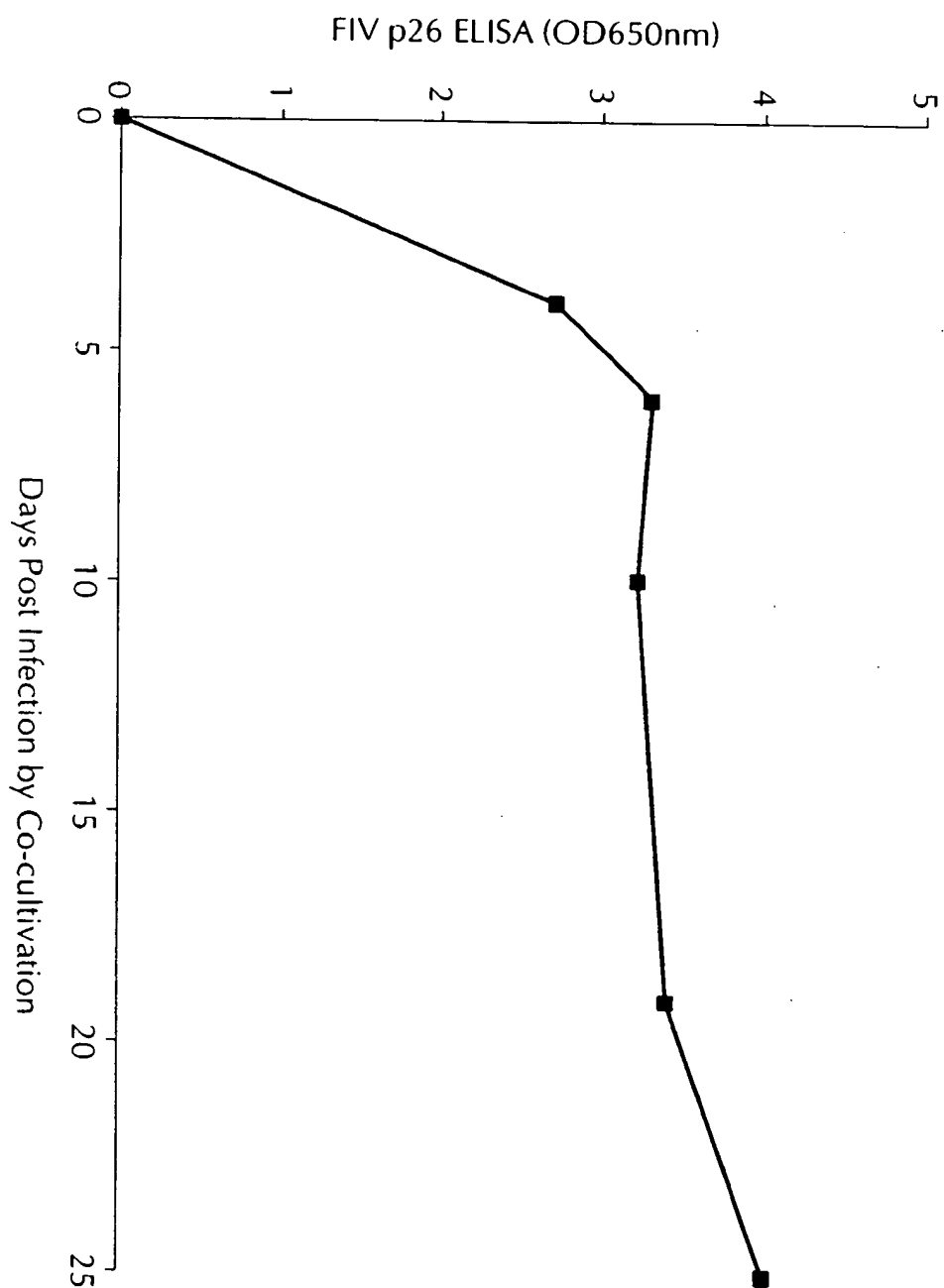
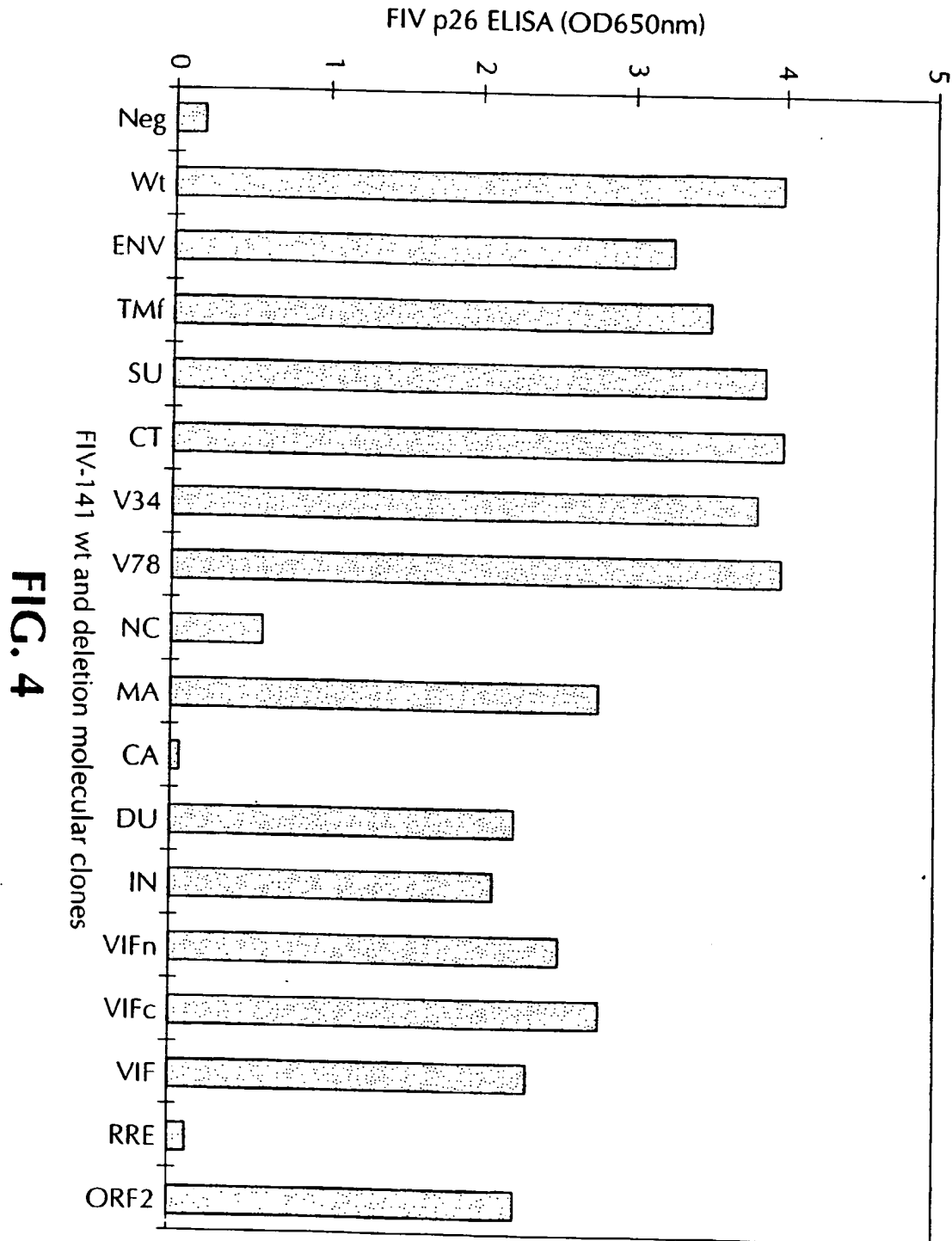


FIG. 2



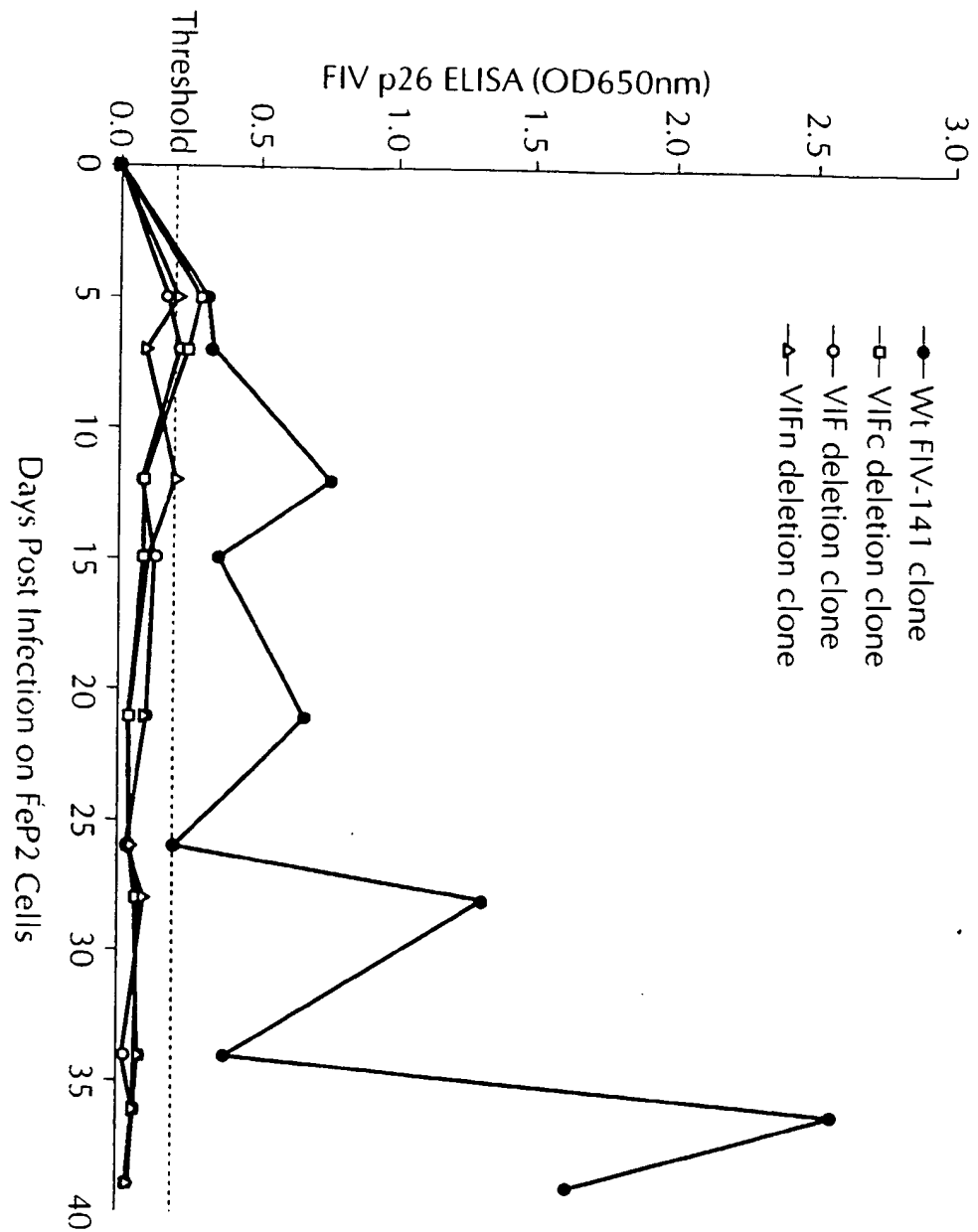


FIG. 6

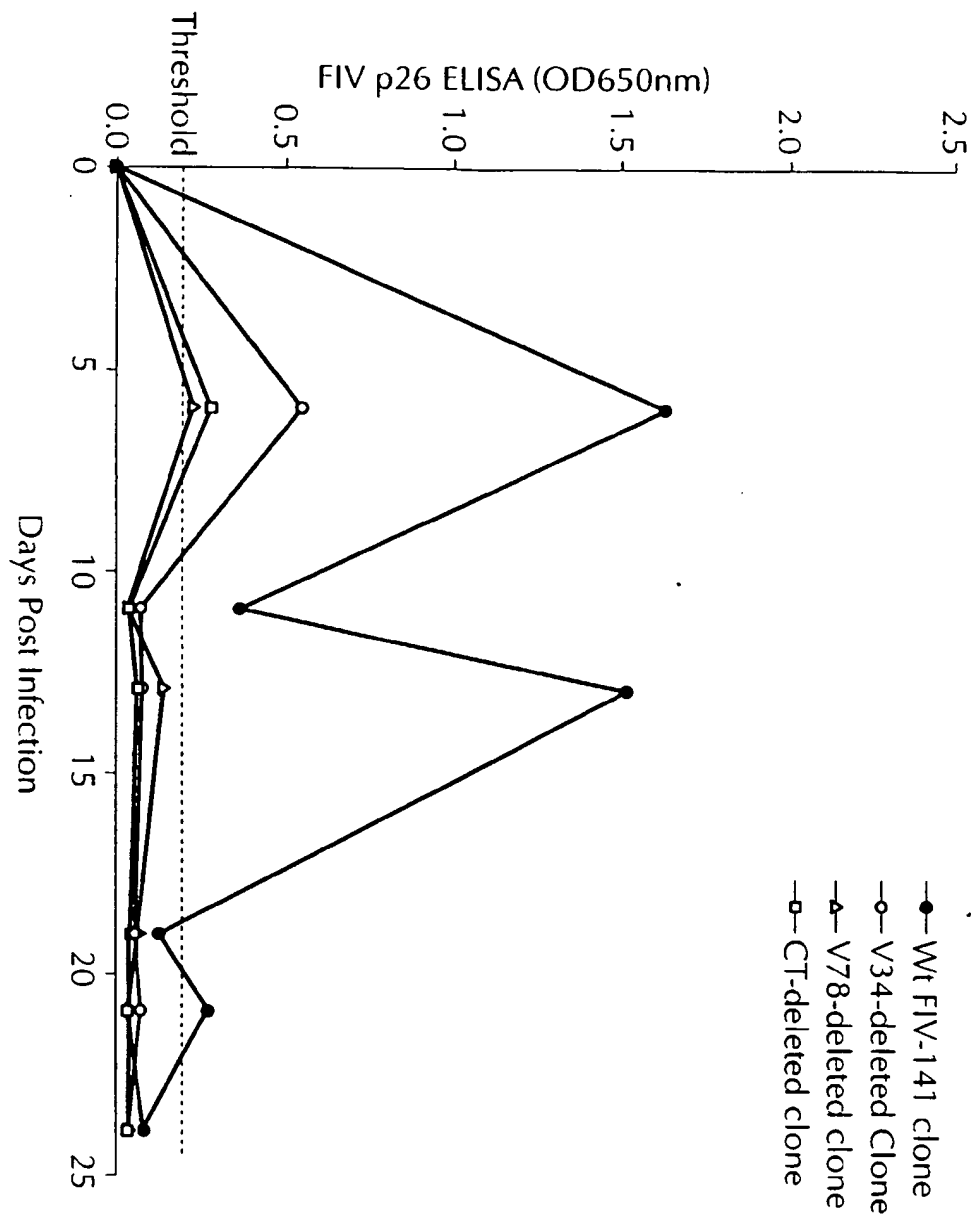


FIG. 8

